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CYP2D6 polymorphisms and the safety and gametocytocidal activity of single dose primaquine for *P. falciparum*

Running title: *CYP2D6* and primaquine safety and efficacy

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Abstract

Single dose primaquine (PQ) clears mature gametocytes and reduces transmission of *Plasmodium falciparum* after artemisinin combination therapy. Genetic variation in *CYP2D6*, the gene producing the drug metabolizing enzyme cytochrome P450 2D6 (CYP2D6), influences plasma concentrations of PQ and its metabolites and is associated with PQ treatment failure in *P. vivax*. Using blood and saliva samples of varying quantity and quality from 8 clinical trials across Africa, (n=1076), we were able to genotype *CYP2D6* for 774 samples (72%). We determined whether genetic variation in *CYP2D6* has implications for PQ efficacy in individuals with gametocytes at the time of PQ administration (n=554) and for safety in Glucose-6-phosphate dehydrogenase (G6PD) deficient individuals treated with PQ (n=110). Individuals with genetically inferred CYP2D6 poor/intermediate metabolizer status had higher gametocyte prevalence on day 7 or 10 post PQ compared to those with extensive/ultrarapid CYP2D6 metabolizer status (OR = 1.79 [1.10, 2.90], p = 0.018). Mean minimum haemoglobin concentration during follow-up for G6PD deficient individuals was 11.8 g/dL for CYP2D6 extensive/ultrarapid metabolizers and 12.1 g/dL for CYP2D6 poor/intermediate metabolizers (p = 0. 803). CYP2D6 genetically inferred metabolizer status was also not associated with anaemia following PQ treatment. (p = 0.331). We conclude that CYP2D6 poor/intermediate metabolizer status may be associated with prolonged gametocyte carriage after treatment with single low dose PQ but not with treatment safety.

Introduction

With recent successes in malaria control and the move towards *P. falciparum* elimination, there is an increasing interest in transmission-reducing strategies. One of the tools available is single low dose (0.25 mg/kg) primaquine (PQ) added to artemisinin combination therapy (ACT). PQ is a drug in the class of 8-aminoquinolines that has been on the market for more than 70 years. In recent years, the addition of PQ to ACTs has received considerable interest because of its ability to rapidly clear mature *P. falciparum* gametocytes and reduce the infectious period compared to ACT alone (1-6).

Cytochrome P450 2D6 (CYP2D6) is a human enzyme involved in the metabolization of 20-25% of all prescribed medicines (7-10). Hundreds of different *CYP2D6* alleles have been discovered, some of which influence activity of the produced enzyme (11). Bennett and colleagues first associated genetic *CYP2D6* variation with relapses of *P. vivax* malaria after PQ treatment (12). More recently, genetic *CYP2D6* variation was found to be strongly associated with an increased risk of relapses among Indonesian patients with clinical *P. vivax* malaria (7). There is also evidence in mice that enzymes in the CYP2D family produce the active metabolite of PQ against *P. berghei* liver stages (13), but metabolic activation of PQ may not be necessary to eradicate blood stages (14).

The implications of genetic *CYP2D6* variation for the use of PQ in *P. falciparum* infections have never been explored. One of the factors that has hindered widespread adoption of PQ for *P. falciparum* transmission-reduction is its safety profile, notably in individuals with genetic deficiencies in glucose-6-phosphate dehydrogenase (G6PD) production (12, 15-17). G6PD is an enzyme involved in the pentose phosphate pathway in human red blood cells (18), and G6PD deficiency (G6PDd) is associated with haemolysis following treatment with PQ. Despite safety concerns related to the haemolytic activity of PQ in individuals with G6PDd, a single low dose of PQ is considered safe in individuals with the most common African G6PDd variant (G6PDd A- variant) (19-21). Since genetic variation in *CYP2D6* influences the pharmacokinetics of single low dose PQ in humans (22), this variation may have implications for PQ efficacy or safety at doses targeting *P. falciparum* gametocytes.

Here, we determine the impact of genetically inferred CYP2D6 metabolizer status on the gametocytocidal and haemolytic effect of single dose PQ in 8 clinical trials conducted across Africa.

Results:

CYP2D6 genotyping with OpenArray technology used here requires high quality DNA, ideally 50 ng/µl, a condition that was not always met. *CYP2D6* genotyping was thus successful in 72% (774/1076) of all samples; success varied considerably between sample types with good success rates for saliva samples (≥98%) and large volume blood samples (≥0.5mL blood; success rate ≥87%) but low success rates for different sample types (1-68%) (Table 1 and Dataset S1). As a result of differences in sample collection methods between sites, genotyping was successful for ≤58% of samples from Uganda and Balonghin, Burkina Faso but for ≥80% of samples for other sites (Table 1 and Dataset S1). Inference of CYP2D6 Activity Score (AS) from genotypes was successful in 68% (731/1076) of samples and presented for the different sites in Figure 1. The CYP2D6 AS inference allowed classification of sample donors as poor metabolizer (PM, activity score = 0), intermediate metabolizer (IM, activity score 0.5-1.0), extensive metabolizer (EM, activity score 1.5-2.0) or ultra-rapid metabolizer (UM, activity score >2.0). For other samples, a range of AS could be inferred that allowed classification into EM/UM classes (AS≥1.5; n=137) (Dataset S2). CYP2D6 PM status was inferred for a minority of individuals (2.6%; 19/731); CYP2D6 IM status was inferred for 38.2% of individuals (279/731).

544 participants from 5 studies who had gametocytes by molecular methods on the day of initiation of treatment, completed treatment, and had complete outcome measures were included in the efficacy analysis. The prevalence of CYP2D6 PM/IM among these individuals was 31.4% (171/544) overall and ranged from 26% to 41% by study. Compared to ACT-treatment alone, PQ was effective in reducing gametocyte prevalence on day 7 or 10 in both CYP2D6 EM/UM (OR = 0.20 [0.11, 0.36], $p < 0.001$) and CYP2D6 PM/IM individuals (OR = 0.15 [0.05, 0.44], $p = 0.001$). Individuals with CYP2D6 PM/IM status had higher gametocyte prevalence at day 7 or 10 post PQ compared to those with CYP2D6 EM/UM status (Table 2), after adjusting for PQ dose, country and baseline gametocyte density (OR = 1.79 (1.10 – 2.90), $p = 0.018$).

For the safety analysis, PQ was administered to 110 G6PDd individuals in 7 different studies. Among these PQ-treated G6PDd individuals, 56% (62/110) were EM/UM and possibly at risk of more severe haemolysis due to increased availability of the active metabolite(s) of PQ. Pre-treatment mean Hb was 13.3 g/dL in the CYP2D6 EM/UM G6PDd individuals and 13.4

g/dL in the CYP2D6 PM/IM G6PDd individuals (p = 0.803). Mean minimum Hb during 10-28 days of follow-up was 11.8 g/dL for CYP2D6 EM/UM G6PDd individuals and 12.1 g/dL for CYP2D6 PM/IM G6PDd individuals. This difference, adjusted for baseline Hb, country and primaquine dose was 0.05 g/dL (95% CI [-0.34, 0.44], p = 0. 803) and not statistically significant. One hundred individuals had Hb measurement on day 7 post-treatment: mean Hb on day 7 was 12.5 g/dL for CYP2D6 EM/UM G6PDd individuals and 12.8 g/dL for CYP2D6 PM/IM G6PDd individuals (adjusted difference 0.25 g/dL; 95% CI [-0.24, 0.74], p = 0.314). 24% (15/62) of CYP2D6 EM/UM G6PDd individuals experienced moderate anaemia compared to 23% (11/48) of CYP2D6 PM/IM G6PDd individuals (adjusted odds ratio 2.11 95% CI [0.46, 9.72], p = 0.334). Only one G6PDd individual from Burkina Faso had severe anaemia post PQ treatment (Hb 7 g/dL at day 10); this individual was CYP2D6 EM/UM, had baseline Hb of 12.5g/dL and recovered completely by day 14 (Hb of 11.9/dL).

Although *CYP2D6* genotyping success was low for some sample sets, it was not associated with persisting gametocytes on day 7 or 10 (OR = 0.95 [0.65-1.38], p = 0.771) or Hb (difference = -0.83 [-1.91, 0.25], p = 0.129) in models adjusted for country, PQ dose and baseline gametocyte density. We thus found no evidence for selection bias in our efficacy and safety outcome assessments due to variation in *CYP2D6* genotyping success.

Discussion

In the current study we utilized samples from clinical trials across Africa to explore the effect of genetically inferred CYP2D6 metabolizer status on PQ efficacy and safety. Compared to ACT alone, the addition of single dose PQ resulted in a marked reduction in gametocyte carriage across populations with different CYP2D6 metabolizer statuses. Nevertheless, CYP2D6 PM/IM individuals were more likely to have persisting gametocytes until day 7 or 10 following initiation of treatment with ACT-PQ.

Whilst the transmission-blocking effect of PQ may precede the gametocyte clearing effect and gametocytes persisting after PQ may not result in onward transmission to mosquitoes (1, 6, 23), the results of the current study suggest that the efficacy of low dose PQ may be affected by CYP2D6 metabolizer status. We previously demonstrated that PQ pharmacokinetics is influenced by genetically inferred CYP2D6 metabolizer status (22), suggesting lower concentrations of the PQ active metabolites may occur in CYP2D6 PM/IM. Whilst CYP2D6 metabolizer status and concentrations of active PQ metabolites have direct implications for *P. vivax*-infected patients by affecting cure rates (12), the effect for *P. falciparum*-infected patients is indirect: potentially increasing the number of secondary cases arising from a PQ-treated gametocyte carrier.

We observed no effect of CYP2D6 metabolizer status on Hb concentrations after PQ treatment of G6PDd individuals. We hypothesized that G6PDd individuals with CYP2D6 PM/IM status would be relatively protected from haemolysis, but this was not observed. Whilst we combined safety studies to maximize the number of observations in G6PDd individuals, it is possible that our study population size was insufficient to detect subtle effects on haemolysis. Inter-study variation may also have obscured effects of CYP2D6 status, although study site was incorporated into our multivariate regression models.

There are several limitations to this study. We worked with available samples from several clinical trials, not specifically collecting material for extensive human genotyping. The variable quality and quantity of samples affected our genotyping success rate but is unlikely to have affected the validity of our comparisons between populations with successful genotyping results. Similarly, the current study did not allow us to detect possible differences in effects between ACTs. CYP2D6 activity and PQ metabolism may be influenced

differently by dihydroartemisinin-piperaquine (DP) (24) and artemether-lumefantrine (AL) (25). Whilst we combined findings from trials with different ACTs, this is unlikely to have affected the validity of our findings and we adjusted for study effects. Another limitation is that we inferred CYP2D6 metabolizer status from the *CYP2D6* genotype. There has been a series of publications describing situations where the commercially available TaqMan assays, also used here in OpenArray format, have not worked as expected, and have been redesigned (26-29). Most significantly, one assay variant detecting CYP SNPs (*15-allele, C__32407245_40) suffers from interference from the sequence of the pseudogene *CYP2D7* to the extent that these results were not included in the analysis (27). Some additional assays have been replaced with new and improved ones during the course of this study (*7-assay C__32388575_30 with C__32388575_A0, *8-assay C_30634117C_20 with C_30634117C_K0, and *14-assay C_30634117D_30 with C_30634117D_M0) (30). In addition, copy number variation (CNV) assay targeting intron 2 (Hs04083572_cn) may not always give the correct result due to intronic polymorphisms and CNV assays in general only work with high sample quality (and not after product pre-amplification). These challenges in genetic analysis underline the complexity of the locus and the need for more sequencing of *CYP2D6*. Especially in African populations for which pharmacogenetic data is lacking, additional data are needed (31). Such future studies may purposefully collect select samples for human genotyping. In our studies 0.5-1mL EDTA blood, or Oragene saliva samples resulted in high genotyping success rates (Table 1). Another option is to perform CYP2D6 phenotyping experiments, where a probe substrate to assess CYP2D6 activity is used. Although substrate specificity may complicate extrapolation of such assays to PQ metabolism, an unquestionable advantage of phenotyping is that it would take into consideration environmental factors influencing CYP2D6 activity. These include, but are not limited to co-morbidities, concomitant medication and food intake (32, 33).

Despite limitations, including the modest number of observations from individuals with the genetically inferred CYP2D6 PM phenotype, we present evidence that CYP2D6 PM/IM status is associated with prolonged gametocyte carriage after treatment. It is currently unclear whether this has implications for the transmission blocking effects of PQ at population level in malaria elimination settings. A clinically meaningful effect of genetically inferred CYP2D6 metabolizer status on PQ-induced haemolysis in G6PDd individuals is unlikely.

Methods:

Study samples

Samples from 8 published clinical trials were used for separate analyses on the impact of genetically inferred CYP2D6 metabolizer status on PQ safety and efficacy. For analyses on the impact of CYP2D6 inferred metabolizer status on PQ efficacy, we included samples from 5 PQ efficacy studies. Gametocyte detection was performed following treatment with a single dose of 0.1-0.75 mg/kg PQ in combination with either artemether-lumefantrine (AL) (Coartem as standard 6-dose regimen over 3 days; Novartis Pharma, Switzerland) in Burkina Faso (3) and Uganda (2), or with dihydroartemisinin-piperaquine (DP) (Eurartesim as standard 3-day regimen; Sigma-Tau, Italy) in Mali (1), The Gambia (4) and Kenya (5). Analyses on the impact of CYP2D6 inferred metabolizer status on haemolysis were restricted to G6PD deficient (G6PDd) individuals; we included two additional studies that specifically assessed PQ safety in G6PD deficient individuals in Mali (20) and The Gambia (19), using 0.25-0.5 mg/kg PQ in combination with DP. In all studies, haemoglobin (Hb) concentration in g/dL was measured by self-calibrating HemoCue photometer (Ängelholm, Sweden). Study details are summarized in Table 1.

Extraction of nucleic acids

An automated MagNA Pure LC 2.0 Instrument (Roche, Switzerland) was used for extraction of Total NA or DNA. For the samples from Uganda as well as the parasitology samples from Mali MagNA Pure LC Total Nucleic Acid Isolation Kit – High Performance, was used. For Burkina Faso, Kenya and samples from the first season of the trial in the Gambia (both full blood in EDTA and saliva samples) MagNA Pure LV DNA isolation kits were used. The saliva samples collected after the second season of the trial in the Gambia were extracted using a MaxWell 16 Instrument (Promega, USA) and Maxwell 16 DNA Purification kits. Concentration measurements were done using a NanoDrop (Thermo Fisher, USA) device (only DNA from full blood in EDTA) and Qubit Fluorometer (Thermo Fisher, USA) with the Qubit HS (High Sensitivity) kit, which is specific for double-stranded DNA (dsDNA).

Gametocyte detection

QT-NASBA was performed as in Schneider et al. (34), qRT-PCR as in Wampfler et al. (35). Briefly, Total NA, was used for amplification of the *P. falciparum* mature gametocyte marker Pfs25 mRNA for the estimation of mature gametocyte density in samples from the clinical trials. Gametocyte densities were assigned based on plate-specific gametocyte dilution series, which was diluted in whole blood before extraction of Total NA, as with the samples from the clinical trials. For samples from trial participants, estimated gametocyte densities below 0.02 gametocytes per µL were considered to be negative.

Ethical considerations

Informed consent was obtained from all study participants. The studies received approval from the Ethics Committee of the Faculty of Medicine, Pharmacy, and Dentistry, University of Science, Techniques and Technologies of Bamako and the Committee on Human Research at the University of California, San Francisco (studies in Mali), Comité d'Ethique pour la Recherche en Santé, Ministère de la Santé du Burkina Faso, Comité Technique d'Examen des Demandes d'Autorisation d'Essais Cliniques, Ministère de la Santé du Burkina Faso (studies in Burkina Faso), The Gambia Government/MRC Joint Ethics Committee (studies in The Gambia), the Makerere University School of Medicine research ethics committee and the Uganda National Council of Science and Technology (study in Uganda), the Kenya Medical Research Institute Ethics Review Committee (study in Kenya) and the Interventions Research Ethics Committee of the London School of Hygiene and Tropical Medicine (all studies).

CYP2D6 metabolizer status

Samples with a sufficient quantities of DNA (50 ng/µl without or 2,5 ng/µl with manufacturer provided pre-amplification kit) were genotyped for *CYP2D6* *2, *3, *4, *6, *7, *8, *9, *10, *11, *15, *17, *18, *19, *20, *29, *40, and *41 alleles using OpenArray technology on a QuantStudio 12K Flex RT PCR system (Life Technologies, Carlsbad, CA, USA). *CYP2D6* copy number was determined with at least one TaqMan copy number assay targeting intron 2 (Hs04083572_cn), intron 6 (Hs04502394_cn) and/or exon 9

(Hs00010001_cn) depending on available sample quality and volume. CYP2D6 metabolizer status was inferred from the genotypes using activity score (AS) (36). An AS of 0.0 = Poor Metabolizer (PM); 0.5-1.0 = Intermediate Metabolizer (IM); 1.5-2.0 = Extensive Metabolizer (EM); and > 2.0 = Ultra-rapid Metabolizer (UM) (37). For the analyses, we compared PM/IM versus EM/UM.

Statistical analysis

As a single measure of PQ efficacy, we used the presence of gametocytes on either day 7 or day 10. The effect on Hb was quantified in two ways: day 7 Hb concentration (from studies with day 7 measurements) and minimum observed Hb concentration (from all studies; up to day 28 after initiation of treatment). Because different trials used different PQ doses, PQ dose was categorized as no PQ (control arms), 0.25 mg/kg PQ (0.10-0.25 mg/kg), 0.5 mg/kg PQ (0.4-0.5mg/kg) or 0.75mg/kg PQ. Anaemia was defined based on criteria of the World Health Organization (38); moderate anaemia was defined as Hb < 11 g/dL for adults or <10g/dL for children <5 years of age; severe anaemia was defined as Hb < 8 g/dL for adults and <7g/dL for children < 5 years of age. Logistic and linear regression models were used to analyse the effect of CYP2D6 status on gametocyte prevalence, anaemia and Hb concentration. Models controlled for PQ dose, study, baseline gametocyte and asexual parasite density (in efficacy analyses), and baseline Hb (in safety analyses).

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288 **Table 1. Trial details and samples available. AL=artemether-lumefantrine, DP=dihydroartemisinin-piperaquine.**

Country, study type	**Falciparum Malaria Status**	**G6PDd Status**	**ACT**	**PQ**	**PQ**	**Days gametocyte measurement**	**Days haemoglobin measurement**	**CYP2D6**		**Samples included**	
				timing, day	**dose (mg/kg)**			**Sample type, n**	**genotyping success, % (n)**	**Efficacy, n**	**Safety, n**
Uganda, efficacy (2)	Uncomplicated malaria	Normal by fluorescent spot test	AL	2	0.75 0.4 0.1	0, 2, 3, 7, 10, and 14	0, 1, 2, 3, 7, 10, 14, 21	50µL EDTA blood in L6 (n=345); filter paper (n=45)	58% blood in L6 (n=226); filter paper (n=2)	138	11
Burkina Faso (Balonghin), efficacy (3)	Asymptomatic infection	Normal by rapid diagnostic test	AL	2	0.4 0.25	0 and 7	0, 1, 2, 3, 7, 10, 14	100µL EDTA blood in RNAprotect (n=100); 0.5-1mL EDTA blood (n=112), Oragene saliva samples (n=27)	57% blood in RNAprotect (n=1) EDTA blood (n=109) Oragene saliva samples (n=27)	182	8
Burkina Faso (Banfora), safety (19)	Asymptomatic infection	Deficient by by fluorescent spot test (and controls)	AL	0	0.4 0.25	0, 3 and 7	0, 1, 2, 3, 4, 5, 7, 10, 14, 28	0.5-1mL EDTA blood (n=78)	97% (n=76)	0	43
Kenya, efficacy (5)	Asymptomatic gametocyte	Regardless of G6PD status	DP	2	0.25	0, 2, 3, 7, and 14	0, 2, 3, 7, 14	0.5-1mL EDTA blood	87% (n=103)	99	7

	carrier							(n=118)			
Mali, efficacy (1)	Asymptomatic gametocyte carrier	Normal by colorimetric quantification	DP	0	0.5 0.25 0.125 0.0625	0, 2, 3, 7, 14, and 28	0, 1, 2, 3, 7, 14, 28	50µL EDTA blood in L6 (n=47); Blood pellets (n=33)	80% Blood in L6 (n=32) Blood pellets (n=32)	56	4
Mali, safety (20)	Parasite free (by microscopy)	Normal controls and deficient by rapid diagnostic test	None	0	0.5 0.45 0.4	None	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 28	0.5-1mL EDTA blood (n=28)	93% (n=26)	0	18
The Gambia efficacy, (4)	Asymptomatic infection	Normal by fluorescent spot test	DP	2	0.75 0.4 0.2	0, 3, 7, 10, and 14	0, 1, 2, 3, 7, 10, 14, 21, 28, 35, 42	Oragene saliva samples (n=85)	99% (n=84)	69	0
The Gambia, safety (19)	Regardless of infection status	Deficient by fluorescent spot test (and controls)	DP	0	0.4 0.25	0, 3 and 7	0, 1, 2, 3, 4, 5, 7, 10, 14, 28	0.5-1mL EDTA blood (n=58)	97% (n=56)	0	19

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290 **Table 2. The effect of CYP2D6 metabolizer status and covariates on gametocyte**

291 **prevalence at day 7 or 10 among individuals receiving primaquine**

	Gametocytes on day 7 or 10 / Total (%)	OR (95% CI)	Adjusted OR[1] (95% CI)
CYP2D6 status:		p = 0.028	P = 0.018
EM/UM	80/289 (28%)	1	1
PM/IM	51/133 (38%)	1.62 (1.05 – 2.50)	1.79 (1.10 – 2.90)
Baseline gametocyte density per ml		1.002 (1.000 – 1.003) p = 0.005	1.002 (1.001 – 1.003) p = 0.004
Baseline asexual parasite density per ml		0.998 (0.994, 1.002) p = 0.380	0.994 (0.982, 0.999) p = 0.025
PQ dose:		p < 0.001	p < 0.001
0.25 mg/kg	89/228 (39%)	1	1
0.5 mg/kg	30/153 (20%)	0.38 (0.24 – 0.62)	0.32 (0.18 – 0.56)
0.75 mg/kg	12/41 (29%)	0.65 (0.31 – 1.33)	0.34 (0.14 – 0.82)
Country:		p < 0.001	p < 0.001
Burkina Faso	26/166 (16%)	1	1
Kenya	20/50 (40%)	3.59 (1.78 – 7.26)	2.24 (1.07 – 4.74)
Mali	21/43 (49%)	5.14 (2.48 – 10.66)	6.05 (2.76 – 13.25)
Gambia	24/61 (39%)	3.49 (1.80 – 6.78)	3.27 (1.62 – 6.59)
Uganda	40/102 (39%)	3.47 (1.95 – 6.19)	4.19 (2.03 – 8.64)

292 [1] Adjusted for all other factors in the table

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Figure legends

Figure 1. Genotypically inferred CYP2D6 AS for six African populations. Only samples for which an exact AS was inferred were included in this figure (n/N where n=number for whom AS was determined and N=number of samples that genotyping was attempted for). It was not possible to infer AS for all with determined genotype due to not knowing which haplotype is duplicated (Dataset S1). In some cases, it was possible to determine an AS range (Dataset S2) but not an exact AS. For Mali and The Gambia results from efficacy and safety studies were combined; in Burkina Faso the efficacy and safety studies were carried out in two distinct populations in different areas and therefore the AS results are presented separately for the Balonghin and Banfora populations.

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